

The evolutionary terrestrialization of planarian flatworms (Platyhelminthes, Tricladida, Geoplanidae): a review and research programme

Ronald Sluys¹

¹ *Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands*

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Corresponding author: *Ronald Sluys* (Ronald.Sluys@naturalis.nl)

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Abstract

The terrestrialization of animal life from aquatic ancestors is a key transition during the history of life. Planarian flatworms form an ideal group of model organisms to study this colonization of the land because they have freshwater, marine, and terrestrial representatives. The widespread occurrence of terrestrial flatworms is a testament to their remarkable success occupying a new niche on land. This lineage of terrestrial worms provides a unique glimpse of an evolutionary pathway by which a group of early divergent aquatic, invertebrate metazoans has moved onto land. Land flatworms are among the first groups of animals to have evolved terrestrial adaptations and to have extensively radiated. Study of this terrestrialization process and the anatomical key innovations facilitating their colonization of the land will contribute greatly to our understanding of this important step in Metazoan history. The context and scientific background are reviewed regarding the evolutionary terrestrialization of land flatworms. Furthermore, a framework of a research programme is sketched, which has as its main objective to test hypotheses on the evolution of land planarians, specifically whether particular anatomical and physiological key innovations have contributed to their evolutionary successful terrestrial colonization and radiation. In this context special attention is paid to the respiration in aquatic and terrestrial planarians. The research programme depends on a comprehensive phylogenetic analysis of all major taxa of the land flatworms on the basis of both molecular and anatomical data. The data sets should be analyzed phylogenetically with a suite of phylogenetic inference methods. Building on such robust reconstructions, it will be possible to study associations between key innovations and the evolutionary terrestrialization process.

Key Words

adaptations, evolution, key innovations, land flatworms, model organisms, respiration, terrestrialization

Introduction

Charles Darwin was fascinated by planarian flatworms, and he was particularly struck by the fact that there is a group of planarians that actually live on land. As he wrote in a letter from 23 July 1832 to his mentor Henslow: “Amongst the lower animals, nothing has so much interested me as finding 2 species of elegantly coloured true Planariae inhabiting the dry forest.” And in a letter from 15 August 1832: “I have today to my astonishment found 2 Planariae living under dry stones....”. Darwin thought for a long time that he was the first person to

have discovered terrestrial flatworms. It was only in 1846 that it came to his attention that already in 1774 the Danish naturalist O. F. Müller had described the land flatworm *Microplana terrestris* (Müller, 1774) (Porter and Graham 2016).

Darwin (1983: 25) appropriately and succinctly described land flatworms as follows: “In general form they resemble little slugs, but are very much narrower in proportion, and several of the species are beautifully coloured with longitudinal stripes.” He took some of

the land flatworms that he had collected in Tasmania on board of the *Beagle* and managed to keep them alive for two months. He experimented on the animals by cutting some animals into half, and he discovered that after 25 days each piece had regenerated to an almost complete animal (Darwin 1983; Sluys 2016). Darwin was so much intrigued by his flatworm findings that in a letter of 22 May 1833, written when he was in Tierra del Fuego, he asked his sister Catherine to send him Dalyell's (1814) paper on several species of British freshwater planarians (Thomson 2009). In this paper Dalyell (1814) reported many observations on the biology of these worms, including a detailed study of their fission and regeneration (Ball and Reyoldson 1981). Since the days of Darwin and Dalyell our knowledge on the regenerative capacity of land planarians, and that of triclad flatworms in general, has greatly increased (cf. Reddien 2018; Rink 2018), as well as our knowledge on the morphology, taxonomy, phylogeny, and distribution of land flatworms (cf. Sluys and Riutort 2018 and references therein). The fact that delicate organisms such as free-living flatworms, most of which occur in freshwater or marine environments, are able to survive on land has received little attention. Thus far, no detailed studies have been undertaken that attempted to analyze how and when during their evolutionary history planarians conquered the terrestrial environment, which of their anatomical and physiological features enabled their colonization of the land, and which adaptations currently facilitate their occupation of the terrestrial niche.

Therefore, in the following I do not so much present the results of such studies, but provide a review of this subject and sketch the context, scientific background, and framework of a research programme in which land flatworms form the model group through which we may not only learn about their own terrestrialization but may be enlightened also on the early evolutionary terrestrialization of animal life in general. In this context, special attention is paid to the respiration in aquatic and terrestrial planarians. In addition, the results obtained during this putative research programme will also provide data for some collateral topics, such as biodiversity assessment and historical biogeography.

Early evolutionary terrestrialization of animal life, as exemplified by land flatworms

The terrestrialization of animal life from marine or freshwater ancestors is a key event in the history of life on earth, particularly because in the course of evolution "...transitions among physically different habitats... are rare" (Vermeij and Dudley 2000: 546; see also Vermeij 2010). Moving from an aquatic to a terrestrial niche is challenging due to the dramatically different demands each environment places on the physiology and structure of an organism, thus requiring numerous innovations.

Key innovations afford enhanced performance, promoting ecological opportunity. These innovations enable subsequent species diversification and radiation. For example, marine gastropod snails in several cases evolved terrestriality independently from amphibious ancestors by side-stepping a major constraint on land snail evolution, viz., the need to produce mucus for locomotion (Rosenberg 1996). Interestingly, mucus plays an important role in land flatworms: forming a slime trail for locomotion, slime threads by which they can cross spaces or lower themselves from heights, and a protective coat against drying. Apparently, the worms have followed a different adaptive pathway than the molluscs and were able to colonize the land without conserving mucus.

The land flatworms or planarians (Fig. 1) likely represent one of the first groups of animals that during evolution have colonized the land and have extensively radiated (see below: Impact and innovative aspects). Study of the evolutionary terrestrialization of the land flatworms and the key anatomical innovations facilitating this process will contribute greatly to our understanding of the early steps onto land of the Metazoa. Planarian flatworms (Platyhelminthes Claus, 1887, Tricladida Lang, 1884) form an ideal group of model organisms to study this process because they have freshwater, marine, and terrestrial representatives. Preadaptations for life on land are the conditions that (1) flatworms have internal fertilization, (2) the cocoons enveloping the eggs have a relatively hard shell, (3) the young hatch directly as young worms and not as larvae (Little 1983), and that (4) the worms produce mucus. Traditionally, three major groups of triclads were recognized: Paludicola Hallez, 1892 (freshwater planarians), Maricola Hallez, 1892 (marine triclads), Terricola Hallez, 1892 (land planarians). A fourth clade, the Cavernicola Sluys, 1990, was proposed by Sluys (1990). More recently, molecular phylogenetic studies have shown that one of the three paludicolan families is more closely related to the land planarians than to the other freshwater planarians (Baguña and Riutort 2004; Álvarez-Presas et al. 2008; see also Sluys and Riutort 2018).



Figure 1. Photograph of the South American land planarian *Polycladus gayi* (from Grau and Carbayo 2010).

Model organism: land planarians

Terrestrial planarians (Platyhelminthes, Tricladida, Geoplanidae Stimpson, 1857) are a relatively species-rich group (approx. 910 nominal species) with a worldwide, mainly pan-tropical, distribution (Fig. 2). The animals live typically in tropical jungles and wooded areas. Terrestrial planarians colonized the land hundreds of millions of years ago, but they still lack any special mechanism for water conservation (Kawaguti 1932). Although they require a humid environment, they cannot endure long submersion in water, in contrast to their marine and freshwater relatives (Froehlich 1955). The animals cannot endure heat and direct sunlight, otherwise they desiccate quickly (Kawaguti 1932). Because of these limitations they tend to remain hidden during the day in humid, but not wet, refuges, only emerging at night when the relative humidity of the air is high. Land planarians are fully terrestrial cryptozoic organisms because they are not tied to aquatic systems for reproduction. The worms are part of the soil ecosystem, living within the habitat of their prey (Ogren 1955; Ball and Sluys 1990; Ogren and Sheldon 1991). Terrestrial planarians are successful top-predators of other invertebrates such as snails, slugs, earthworms, isopods, insect larvae, and springtails; they themselves are rarely predated upon by other organisms, although some of their predators may be beetles, snails, and other land planarians (Boll and Leal-Zanchet 2018 and references therein). Land planarians search for, attack, and capture prey much larger than themselves, employing various techniques such as physical force, adhesive mucus, pharyngeal action, and pouring very effective digestive secretion over the surface of the live prey, or into it, by the protrusible pharynx.

The widespread occurrence of these terrestrial flatworms (Fig. 2) is a testament to their remarkable success occupying a new niche on land for hundreds of millions of years. This lineage of flatworms thus provides a unique glimpse of an evolutionary pathway by which a group of early divergent aquatic, invertebrate metazoans has moved onto land.

Objectives

The main objective of the research programme described here is to test hypotheses on the evolution of land flatworms, specifically whether particular anatomical and physiological key innovations have contributed to their successful terrestrial colonization and subsequent radiation (see below: Hypotheses testing). This first requires a comprehensive phylogenetic analysis of all major taxa of the land flatworms (e.g., for the current 55 genera; cf. Sluys et al. 2009). The project needs to construct this phylogeny on the basis of both molecular and anatomical data. This reconstruction forms the necessary basis and framework for subsequent comparative studies of key innovations, adaptive radiation, and historical biogeography of land flatworms, and on the early terrestrialization of animal life.

Key innovations, adaptive radiation, and terrestrialization

Understanding of the evolutionary dynamics of the following presumed key innovations, for example, may contribute to our insight in the evolution of land flatworms: (1) colonization of the land (on which occasions did the transition from water to land occur and were there reversals?); (2) contribution of the various kinds of anatomically complex creeping soles and (3) of mesenchymal body musculature (absent in freshwater and marine forms) to the effective terrestrialization; (4) contribution of cephalic specializations for the capture of prey to the adaptive radiation process; (5) the relation between the ecology and anatomy of the various taxa and their various types of pharynges (frequently totally different from freshwater and marine forms) for capturing and digesting prey; (6) the extent to which the various kinds of multi-cellular eyes of land flatworms (completely different from marine and freshwater forms) facilitated terrestrialization and adaptive radiation; (7) adaptation of particular sense organs, such as olfactory chemoreceptors, to the humid air of the terrestrial environment, in contrast to taste chemoreceptors that evolved in aquatic habitats; (8) the way in which the worms are able to cope with a major evolutionary constraint: the need to produce mucus for their locomotion, mucus for the most part being water; (9) the correlation between various body shapes (cylindrical, flat, etc.) on the one hand and water conservation and various terrestrial habitats (ranging from humid to rather dry) on the other hand, a cylindrical body considered to be more economical in terms of water conservation (Clark and Cowey 1958); (10) the manner in which the protonephridia adapted from an osmoregulatory system in aquatic ancestors to a resorptive system (saving water and/or eliminating metabolic wastes) in land flatworms; (11) the change from aquatic to terrestrial respiration.

Impact and innovative aspects

Phylogenetic studies, including those on flatworms in general and land flatworms in particular, are generally based on one-sided approaches, incorporating either morphological/anatomical data, or molecular data. Molecular studies might plot some morphological data on the resulting phylogenies to legitimize the molecular trees ('pseudo-morphology'; cf. Mooi and Gill 2010; Assis and Rieppel 2011; see also Williams and Ebach 2010). But for the land flatworms there are no studies that take an integrative approach with both kinds of data. This contrasts with the fact that many interesting scientific questions in this group of animals concern the evolution of their structures, key innovations, and adaptations (see above: Key innovations, adaptive radiation, and terrestrialization).

Land flatworms likely are among the first groups of animals that during evolution have colonized the land

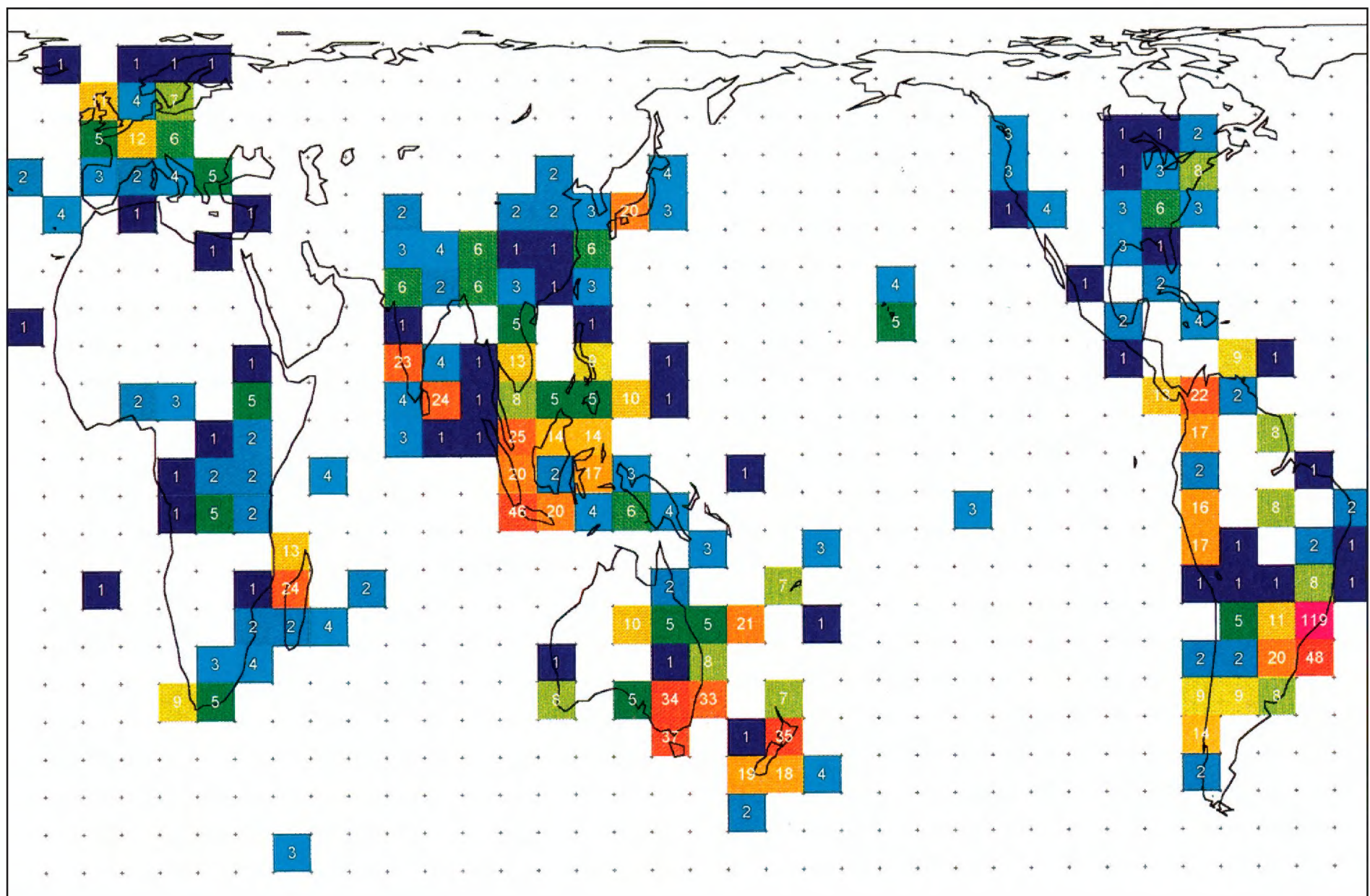


Figure 2. Map of species richness in land planarians on an equal area grid map; maximum in red, minimum in dark blue (from Sluys 1998). Over the past 20 years new discoveries have added to the number of native and introduced species. Nevertheless, the current pattern of biodiversity remains very similar to the one shown in this map, although now, for example, about 20 new species are recognized in Europe, 40 in southeastern and southern Brazil and northeastern Argentina, nine in the Australian territories, and six in New Zealand (Sluys 2016).

and have subsequently radiated extensively. According to fossil information arthropods would be the first animals to have colonized the land, with atmospheric oxygen levels as the major driver of successful colonization (Ward et al. 2006). However, phylogenetic trees suggest flatworms as one of the early colonizers (cf. Hedges and Kumar 2009). This is generally neglected and, therefore, the study proposed here will contribute to a more balanced representation of the evolutionary history of terrestrial animal life.

The fossil record of flatworms is sparse and hardly provides adequate calibration points for a molecular clock (cf. Pierce 1960; Alessandrello et al. 1988; Ruiz and Lindberg 1989; Poinar 2003); calibration points may have to be based on paleogeographical information, amber fossils, coprolites, or estimates of mutation rates (cf. Blaxter 2009 and references therein). Thus, the proposed project will also form a first step towards our understanding of the absolute timing of the early terrestrialization of animal life. It will facilitate the test of the hypothesis that terrestrialization is linked to atmospheric oxygen levels, as was proposed for arthropods (Ward et al. 2006). The project may thus contribute to the first few insights into the terrestrialization of the Lophotrochozoa Halanych et al., 1995 because our current understanding is mainly re-

stricted to the Ecdysozoa Aguinaldo et al., 1997 and the Deuterostomia Grobden, 1908 (cf. Labandeira 2005). Such issues may only be adequately addressed with the help of the phylogenetic trees generated during the research programme outlined here. These phylogenetic reconstructions will also form the long-awaited robust backbone for conservation biology studies in which land planarians function as indicator taxa (see Sluys 1999). Furthermore, triclad flatworms are a key group in historical biogeography because they do not possess larval dispersive stages. Therefore, they are excellent models for vicariance scenarios as explanations for current biogeographic patterns. Thus, the research programme will facilitate tests of the hypothesis that plate tectonics has been a major factor in their historical biogeography (cf. Sluys 1994, 1995).

For the morphological data I envision this programme to develop a formal knowledge representation (ontology) of planarian phenotypes and character states that leverages progress in this field (for a review, see Mabee et al. 2007) such that one of the outcomes will be a ‘Rosetta stone’ of concepts in planarian morphology that can be used not just to disambiguate characters and their states in the putative study but also to inform subsequent research questions in connecting genomic data and planarian phenotypes.

Phylogeny

Knowledge on the phylogenetic relationships within the Tricladida is based on phylogenetic analyses of molecular and morphological datasets (for a review, see Sluys and Riutort 2018). Attention has been paid mostly to: (1) the relationships between the higher taxa within the Tricladida (cf. Sluys 1989a; Carranza et al. 1998; Álvarez-Presas et al. 2008); (2) the affinities within the suborder of the Maricola (Sluys 1989b); (3) the higher taxon relationships within the freshwater planarians in general and within the family Dugesiidae Ball, 1974 in particular (Ball 1974; De Vries and Sluys 1991; Sluys 2001; Lázaro et al. 2009). Sluys and Kawakatsu (2006) explored the phylogenetic relationships within the freshwater families Dendrocoelidae Hallez, 1892 and Kenkiidae Hyman, 1937. More recent studies have resulted in an expanded character state matrix, including several new species and gene sequences, and in a robust phylogenetic tree for the higher taxa (Álvarez-Presas et al. 2008).

For decades the evolutionary relationships within the group of land flatworms have been neglected. Partial taxonomic revisions of the group have been published but these were rarely based on a phylogenetic analysis (cf. Ogren and Sluys 1998; Kawakatsu et al. 2005). It was only with the advent of molecular techniques that studies started to address the phylogenetic structure within the group (cf. Álvarez-Presas et al. 2008; Carbayo et al. 2013). On the basis of the results of these molecular studies, and in combination with anatomical data, a revised higher classification of planarian flatworms was proposed (Sluys et al. 2009), in which the subordinal taxonomic rank of the terrestrial planarians was downscaled to the level of family, viz., the Geoplanidae. However, these molecular studies suffer from the fact that they only incorporate relatively few exemplar species and then only for a small selection of land planarian taxa.

Methodology

Separate and combined analyses

It is proposed here to use both molecular and morphological data sets. To characterize their phylogenetic signal the character state matrices should be analyzed separately, using parsimony (morphology) and Bayesian methods (morphological as well as molecular). In addition, molecular and morphological data sets should be combined into one joint analysis. With respect to morphological and molecular characters two approaches may be followed: (1) morphology is merely optimized in post-tree analysis of the molecular results, or (2) morphological and molecular characters are combined into one data matrix. The first approach is favoured in many recent studies and considered to be the only contribution of morphology to phylogenetic analysis by Scotland et al. (2003); but see Jenner (2004) for a rebuttal. It is here

suggested that the second approach, i.e., combined analysis, is applied.

From an empirical perspective, it has been shown that morphology can have a profound effect on the combined analysis, irrespective of the fact that the number of molecular characters generally exceeds the number of morphological features (Jenner 2004; Assis 2009). Combined analyses may be different from the analyses of the separate data matrices and consensus trees may hide a phylogenetic signal that is generated by a total evidence analysis. Positive contributions of morphology to quantitative clade support measures in combined analyses have been observed for a large number of taxa (Jenner 2004).

Timetree calibration

As the fossil record of flatworms is sparse and does not provide adequate calibration points for a molecular clock, calibration of the phylogenetic timetrees has to be based on other kinds of data, such as, for example, paleogeographical information (see above: Impact and innovative aspects). This means, for example, that we will be looking for closely related taxa **a** and **b** that are endemic to the areas **A** and **B**, respectively. In addition, we will be looking for those areas **A** and **B** inhabited by endemic taxa for which paleogeographic data indicate the time since the two areas have fragmented from a single ancestral area. These two pieces of information, together with the molecular clock hypothesis, will enable one to date all cladogenetic and biogeographic events in the entire lineage of which **a** and **b** only form a part.

Within the triclad flatworms there is a good number of such disjunctions, due to vicariance events, within species or between closely related species, that may be tested as possible calibration points. For example, land planarians of the genus *Othelosoma* Gray, 1869 are restricted to Africa and India and have attained their current distribution when India and Africa started to separate at about 150 Mya. Table 1 specifies the taxa, and their presumed vicariance events and divergence times that may profitably be used for calibration.

One may perhaps be inclined to consider paleogeographical calibrations to be less ideal than fossil calibrations. In point of fact, the opposite may be the case. Fossil-calibrated molecular clocks at best provide minimum dates (Wilke et al. 2009). Therefore, it has been argued that clocks are best calibrated with reference to the distribution of molecular clades and associated tectonics (e.g., Azuma et al. 2008; Heads 2014).

Molecular dating is a rapidly developing field and therefore there is currently no single best method; each approach has its advantages and disadvantages. It is also important to note that in the phylogenetic tree of the Platyhelminthes, the triclads constitute one of the crown groups (cf. Baguña and Riutort 2004). This implies that the nodes below the Tricladida represent older taxa that therefore might be used as potential outgroups in the phylogenetic analysis.

Datasets and phylogenetic analysis

The molecular data matrix for this project may be derived from published data from GenBank, and new molecular sequences generated from fresh material of new taxa examined during the project. Previous studies have shown that the following genes provide the best resolution at the hierarchical levels of the phylogenetic tree of the triclads that form the focus of the putative research programme: nuclear 18S rDNA and 28S rDNA for resolving the deeper, more ancient branches in the tree, and mitochondrial COI to contribute signal on more recent splits (e.g. between and within genera) (Álvarez-Presas et al. 2008). Subsequently, another nuclear marker, the elongation factor 1-alpha (EF), was added to the list of informative markers for an intermediate level between family and species, contributing to resolution of the relationships between members of the Geoplaninae subfamily (Carbayo et al. 2013).

It has been suggested that many of the problems associated with the amplification and sequencing of planarian molecular markers may be solved by applying next generation sequencing (NGS) methodologies (Sluys and Riutort 2018). This new era has started already, and in recent years genomes and transcriptomes of freshwater planarians have already been sequenced (Egger et al. 2015; An et al. 2018; Grohme et al. 2018; Rozanski et al. 2018), contributing new anonymous nuclear markers that can be used as phylogenetic tools. There are even bioinformatic pipelines that can help in the discovery of these markers using NGS data as input (Frías-López et al. 2016). This has already been applied in freshwater planarians, and will soon be used also for terrestrial flatworms. Moreover, there is now available also information on other mitochondrial markers, in addition to COI, as whole mitogenomes were sequenced and annotated (Sakai and Sakaizumi 2012; Solà et al. 2015; Gastineau et al. 2019; Yang et al. 2019), contributing to the combination of nuclear and mitochondrial markers, thus obtaining better resolved phylogenies.

The project requires a large morphological data matrix of all major taxa of land planarians (e.g., for the current 55 genera; cf. Sluys et al. 2009). For this, the programme may be taking as leads the doctoral dissertations of both Dr L. Winsor (2003, James Cook University, Townsville)

and Dr F. Carbayo (2003, University of Sao Paulo). Both researchers made detailed character codings and scorings for taxa of land planarians. Dr Winsor mostly focussed on Australian taxa, while Dr Carbayo concentrated on South American species, although neither used these scorings for a subsequent phylogenetic analysis. During the project character codings and scorings may be extracted from these two doctoral theses and, subsequently, be combined, refined and also supplemented by scorings for taxa not examined by Winsor or Carbayo. Definition and scoring of morphological character states should comply with the most recent insights (cf. Sereno 2007).

The morphological, molecular, and combined data matrix may be analyzed using maximum parsimony (e.g., TNT; Goloboff et al. 2003), maximum likelihood (e.g., RaxML; Stamatakis 2014; IQtree; Nguyen et al. 2015), and Bayesian Inference (MrBAYES, Ronquist et al. 2012, and BEAST, Drummond et al. 2012). Informative genes should first be analyzed separately, but also combined in the form of concatenated datasets. Substitution model parameters may either be selected using nested likelihood ratio tests or explored using reversible jump Markov chain Monte Carlo methods (jModeltest, Darriba et al. 2012; Partitionfinder, Lanfear et al. 2017). Saturation tests should be performed using DAMBE (Xia 2017).

Molecular data may be analyzed also under a dynamic approach to homology. In the dynamic approach delineations are dependent upon the topology of the phylogenetic trees on which they are optimized. In this context hypotheses of homology are part of phylogenetic hypotheses and are subject to the same optimality criteria as the trees, viz., minimisation of evolutionary transformation events. The computer program POY (ver. 4.0 beta 2635; Wheeler et al. 2006, Varón et al. 2010) allows for phylogenetic tree searches under this dynamic homology or direct optimization approach.

Aquatic planarians should form one of the outgroups for the phylogenetic analysis. Particularly the freshwater family Dugesiidae has been shown to share a close relationship with the land flatworms (see Sluys et al. 2009). But other freshwater groups, as well as marine triclads, should be included also as outgroups. Comparison with these freshwater and marine forms will reveal the adaptations that made possible the transition to land.

Table 1. Taxa, and their presumed vicariance events and divergence times that may be used for calibration of the timetrees.

Taxon	Vicariant distribution	Divergence time (Mya)
<i>Othelosoma</i> species	Africa (31 sp.)/India (7 sp.)	≤150
<i>Bipalium</i> species	Madagascar (23 sp.)/India & SE Asia (160 sp.)	90-50
<i>Girardia</i> species	N. America (4 sp.)/ S. America (39 sp.)	3,5
genus <i>Girardia</i> /genus <i>Dugesia</i>	N. & S. America (42 sp.)/Africa (21 sp.)	130–100
<i>Dugesia</i> species	E. Med. (12 sp.)/W. Mediterranean (10 sp.)	38-3
<i>Procerodes littoralis</i>	E. Atlantic/W. Atlantic	150
<i>Foviella affinis</i>	E. Atlantic/W. Atlantic	150
<i>Uteriporus vulgaris</i>	E. Atlantic/W. Atlantic	150
genus <i>Amblyplana</i> /genus <i>Geoplana</i>	Africa (9 sp.)/S. America (64 sp.)	100
<i>Romankenkius</i> species	S. South America (1 sp.)/Australia (12 sp.)	120

Hypotheses testing

Creeping sole

The research proposed here seeks to explore the evolution of terrestrialization of land flatworms in time and in correlation with presumed key morphological adaptations. As regards the time axis, this will be important both for direct reconstructions of when terrestrialization happened, as well as in the subsequent analysis of whether hypothesized key innovations co-vary in their location on the phylogeny with elevated diversification rates under a model of adaptive radiation (e.g., using the method of Ree 2005). It is here proposed that divergence dates estimates may be reconstructed from molecular data calibrated using previously published node ages (Hedges and Kumar 2009) in a relaxed molecular clock approach as implemented in BEAST (Drummond et al. 2012). As for covariance between traits (see above: Key innovations, adaptive radiation and terrestrialization), the general model will be that the radiation of flatworms, with presumably repeated terrestrializations, constitutes a natural experiment such that morphological changes hypothesized to be associated with terrestrialization co-vary with and co-occur in ancestral state reconstructions on a phylogenetic tree. Sophisticated comparative methods to correct for autocorrelation (because the different lineages are related to each other) have been developed, including Bayesian statistical methods that allow for phylogenetic uncertainty (e.g., Pagel and Meade 2006). Understanding of the evolutionary dynamics and consequences of such innovations will contribute to the unraveling of their genetic basis and their role in speciation events and, consequently, the adaptive radiation of this group of animals.

To give an example, the study will provide insight into the evolution of creeping soles, the latter defined as: “A flat or ridged modified strip of epithelium on the ventral surface of geoplanid triclad flatworms characterized by the presence of cilia . . . which provides propulsive forces by ciliary or muscular action, or by a combination of both” (Sluys et al. 2009: 1773). Traditionally, a narrow creeping sole is considered as the primitive character state, while medium to broad soles and loss of a creeping sole are seen as derived states. However, narrow soles may also secondarily result from partial reduction, while absence of a creeping sole altogether may be the ancestral state, as aquatic forms lack one. However, the evolution of creeping soles has never been analyzed within a phylogenetic context; the research programme developed here will be the first to do so. If the traditional view holds true, one would expect to infer the narrow creeping sole as the character state at the root of the tree, with transitions to medium or broad soles, or loss of a creeping sole, nearer the tips. Under the opposite scenario, the absence of a creeping sole being the ancestral state, one would consequently expect this state to be reconstructed

for the root of the phylogeny with alternately polarized state changes reconstructed nearer the tips. Likewise, expectations can also be formulated for body shape: if cylindrical body shapes indeed conserve water one would expect transitions to this morphology to coincide with, or follow terrestrialization when reconstructed on the phylogeny. In a similar way, all of the hypotheses mentioned above (see above: Key innovations, adaptive radiation and terrestrialization), as well as others that may arise during the study, can be tested.

Respiration in planarians

Introduction

A particular crucial feature that may be among the most difficult to examine and to plot its character states on the phylogenetic trees concerns the adaptation to terrestrial respiration. Planarian flatworms possess neither circulatory nor respiratory systems for transporting oxygen or digested food substances to the internal tissues. In these animals, oxygen is absorbed across the entire body wall and for this diffusion process water is required to dissolve oxygen and carbon dioxide in order to cross cell membranes. Clearly, this poses no problem for freshwater and marine planarians as they live in an aquatic habitat, but when ancestral planarians colonized the land, leaving this aquatic milieu must have formed a major hurdle, as the physical properties of water and air are so different. It is true that many land planarians live in habitats with a high humidity, but still these conditions greatly differ from a fully aquatic environment, while there are also terrestrial planarians that occur in mesophile and xerophile habitats (Froehlich 1955; Winsor et al. 1998).

Thickness of flatworms

The analysis of this subject is complicated by the fact that not much is known about the respiratory physiology of free-living flatworms in general and planarians in particular. Furthermore, most of these studies concern aquatic species. A striking example is McNeill Alexander's (1979) conclusion that the maximum possible thickness of a free-living flatworm is 1.0 mm or at most 1.5 mm. His conclusion is based on an argument that involves the calculation of the diffusion rate of oxygen. In philosophical context McNeill Alexander's type of functional explanation has been presented as an example of viability explanations, which are distinct from causal or historical explanations (Wouters 1995).

McNeill Alexander's (1979) calculation is based on a number of factors, viz., (a) partial pressure of oxygen in water, (b) rate of gas diffusion, (c) the fact that oxygen mostly will diffuse through the dorsal body surface, as the ventral surface is in contact with the substrate, (d) the diffusion constant for oxygen diffusing through connective tissue, (e) density of flatworm tissue. When the known or estimated values for these variables are used

in a derivation using Fick’s law of diffusion, “This calculation indicates that the maximum possible thickness for a flatworm ... is about 0.5 mm if oxygen diffuses in only from the dorsal surface, or 1.0 mm if it diffuses equally from the ventral surface.... A similar calculation for a cylindrical turbellarian indicates that the maximum possible diameter would be 1.5 mm This is probably why large flatworms are flat.” (McNeill Alexander 1979: 185).

As flatworms rely entirely on oxygen diffusion through the surface, it is indeed advantageous to have a large surface area:volume ratio, i.e., to be flat and not cylindrical. In general, this holds true for aquatic species, while within species their thickness hardly or not at all increases with an increase in plan area of the body (Callow 1987). Another interpretation of the broad bodies of large land flatworms is that this facilitates capture and subdual of prey (cf. Cseh et al. 2017), i.e., forms an evolutionary adaptation to new kinds of terrestrial prey not encountered by their aquatic ancestors.

For an organism that relies on direct diffusion of oxygen Prosser (1973) provided a formula for the calculation of the thickness of the animal, based on the oxygen concentration of the medium, a diffusion coefficient K, and the rate of oxygen consumption. When the calculation is done for both water and air (Table 2) it reveals that air-breathers can be six times thicker because there is more oxygen in air than in water (it should be noted that there is uncertainty about the units in which thickness is expressed).

Nevertheless, the conclusion of McNeill Alexander (1979), as well as the calculation on which it is based, is open to a number of criticisms. First of all, it is certainly not the case that large flatworms are always very flat or thin. For example, “giant” freshwater species from Lake Baikal, such as *Bdellocephala bathyalis* Timoshkin & Porfirjeva, 1989, are certainly thicker than 0.5–1.0 mm (*B. bathyalis* measuring 3.3 mm in thickness in preserved condition; Sluys et al. 1998). And also the large land planarians are usually thicker, such as *Polycladus gayi* Blanchard, 1845 (4 mm thick), *Pseudogeoplana lumbricoides* (Schirch, 1929) (3.5 mm), *P. nigrofusca* (Darwin, 1844) (3 mm), *Geoplana rufiventris* Schultze & Müller, 1857 (2.3 mm) (Von Graff 1899). Although such measurements on the thickness are usually made on preserved specimens, and that thus fully stretched live specimens will be thinner, this does not necessarily compromise the picture. It should be realized that most of

the time the flatworms hide under stones or fallen logs, etc. and that during this resting period they are highly contracted, thus presumably approaching the condition of preserved specimens.

Another example of a thick flatworm-like animal is the basal bilaterian *Xenoturbella bocki* Westblad, 1950, which may reach a length of 2–3 cm and a thickness of 5 mm (Franzén and Afzelius 1987).

Another objection that may be raised against McNeill Alexander’s (1979) calculation concerns his assumption that diffusion in flatworms is more or less equal to that in frog muscle and connective tissue, i.e., $2 \times 10^{-5} \text{mm}^2 \text{atm}^{-1} \text{s}^{-1}$. However, it is doubtful that the mesenchyme and gut tissue—forming the major component of the planarian body—have the same diffusion constant as frog muscles and connective tissue. Evidently, the diffusion constant of planarian tissue will not be equal to that in water ($6 \times 10^{-5} \text{mm}^2 \text{atm}^{-1} \text{s}^{-1}$) but perhaps $4 \times 10^{-5} \text{mm}^2 \text{atm}^{-1} \text{s}^{-1}$ would be a value that is more realistic for flatworms. This would then imply a higher rate of diffusion and, consequently, would allow the worms to be thicker.

For the rate (*m*) of oxygen consumption per unit volume of tissue, McNeill Alexander (1979: 185) used 0.1 cm³ oxygen g⁻¹h⁻¹, “or a little more”. However, according to Hyman (1951: 207 and references therein) this may be higher, viz., 0.2–0.31 cm³ oxygen g⁻¹h⁻¹, that is “0.2–0.3 cc per gram per hour in adult worms.” According to Moore (2006) it would be about 0.1–0.2 ml oxygen g⁻¹h⁻¹ at 15 °C.

A variable that is not taken into account by McNeill Alexander (1979) is the relationship between size and metabolic rate in flatworms. Although data are scant, generally respiratory rate decreases as size increases (Vernberg 1968).

Although McNeill Alexander’s view on the maximum thickness of flatworm-like animals has been explicitly or implicitly endorsed (e.g., Ruppert et al. 2004; Moore 2006), he considered it merely “a very rough calculation in a textbook” and he conceded that “There are many doubtful assumptions in the calculation” (McNeill Alexander in litt., 22 August 1996).

Respiratory pigments

Apart from the doubtful assumptions in McNeill Alexander’s calculation, another explanation for the empirical fact that flatworms frequently are thicker than 0.5–1.0 mm may lie in the presence of

Table 2. Data and results of thickness calculation according to Prosser’s (1973) formula: thickness = $\sqrt{(8C_o(K/V_{o_2}))}$; oxygen concentration in medium (*C_o*) and K value (for muscle) from Prosser (1973); oxygen consumption (*V_{o₂}*) converted from value of 0.2 ml/g/hr (= 0.0033 ml/g/min; see this paper).

Medium	Oxygen medium (ml/ml)	K (muscle; cm2/min/atm)	Oxygen consumption (ml/g/min)	Thickness (units ?)
Water	0.00651	0.000014	0.0033	0.014864234
Air	0.2095	0.000014	0.0033	0.084322613

respiratory pigments, transporting oxygen across tissues. Hemoglobin is the most common respiratory pigment among invertebrates in general (Brusca and Brusca 2003). With respect to flatworms it has been reported mostly from parasitic helminths (Weber and Vinogradov 2001) as well as for a number of ecto- and endosymbiotic turbellarians, such as *Paravortex* Wahl, 1906, *Triloborhynchus* Bashiruddin & Karling, 1970, *Cleistogamia* Faust, 1924, *Serita* Cannon, 1982, *Paranotothrix* Cannon, 1982 (Jennings and Cannon 1987; Jennings 1988, 1997). Hemoglobin is known also from the free-living flatworms, viz., a species of *Phaenocora* Ehrenberg, 1835 (Vernberg 1968; Weber and Vinogradov 2001). It has been hypothesized that presence of hemoglobin in the entosymbiotic graffiid rhabdocoel *Paravortex scrobiculariae* (Graff, 1882) represents an adaptation to the oxygen-poor conditions to which this species is subjected, in contrast to the well-aerated habitats of other species, such as *P. cardii* (Hallez, 1908) and *Graffilla buccinicola* Jameson, 1897, which lack hemoglobin (Jennings 1981). Unfortunately, oxygen carrying components such as hemoglobin have not been studied in planarians. Winsor (1998) mentioned the possible respiratory role of uroporphyrins (other than hemoglobin) present in the rhabdoids of *Platydemus manokwari* de Beauchamp, 1962. Potential presence of hemoglobin may be deduced from the presence of non-epidermal pigments, often situated in or around specific organs, such as the brain and the pharynx (Jennings 1981, 1988; Jennings and Cannon 1987). In planarians, body colouration generally is due to the presence of granular pigments located in the mesenchyme directly below the dorsal epidermis (Sluys and Riutort 2018), while in most species of the freshwater genus *Girardia* Ball, 1974 the pharynx is also pigmented. Therefore, red pigment at other locations in the planarian body may point to the presence of hemoglobin. However, I have rarely observed pigment to be present at such other locations, at least in histological preparations, suggesting that hemoglobin is absent in triclad flatworms.

Respiration in triclads and other free-living turbellarians

Oxygen consumption in planarians and other free-living turbellarians has been studied chiefly in freshwater species (cf. Vernberg 1968; Heitkamp 1979) and only once in a terrestrial species (Daly and Matthews 1982). An important result that emerged from these studies is the relationship between body size and respiration, in that generally smaller animals have a higher rate of oxygen consumption than larger specimens, when determined on a weight-specific basis; however, some species showed the reverse correlation, while for others no relationship could be established between body size and oxygen uptake (Vernberg 1968 and references therein; Heitkamp 1979).

Rates of oxygen consumption may be determined and expressed in different ways and are generally influenced by the temperature of the habitat. The type of response to changes in temperature varies per species, as some species are eurythermal and others much more stenothermal in their ecological requirements (Vernberg 1968 and references therein). Therefore, respiration rate frequently is expressed as microliters-O₂-per-(milli-)gram-wet weight-per hour ($\mu\text{LO}_2/(\text{m})\text{g}/\text{WW}/\text{h}$) at a particular temperature. Another way to express oxygen consumption is by calculating the coefficient *b*, or the regression slope of a particular power function for the relation between body size and respiration. Unfortunately, the value of *b* varies much as it greatly depends on the size of the animals, their physiological condition, and many external factors (Heitkamp 1979).

For five species of freshwater planarians (*Dugesia gonocephala* (Dugès, 1830), *Crenobia alpina* (Dana, 1766), *Polycelis nigra* (Müller, 1774), *P. felina* (Dalyell, 1814), *Schmidtea polychroa* (Schmidt, 1861)) the following values were found for oxygen consumption ($\mu\text{LO}_2/\text{g}/\text{WW}/\text{h}$), measured at a temperature of about 15 °C: 170, 240, 135, 199, 116, respectively (Vernberg 1968). Unfortunately, these values for triclad flatworms cannot be compared directly with those determined for various microturbellarians as these were calculated per milligram wet weight ($\mu\text{LO}_2/\text{mg}/\text{WW}/\text{h}$), resulting in the following values, measured at a temperature of 15 °C: 1.242 (*Dalyellia viridis* (Shaw, 1791)), 0.688 (*Opisthomum pallidum* Schmidt, 1848), 0.168 (*Mesostoma ehrenbergi* (Focke, 1836)), ranging between 0.298 and 0.700 (eight different populations of *Mesostoma lingua* (Abildgaard, 1789)) (Heitkamp 1979).

The habitat temperature of the tropical terrestrial land planarian *Bipalium kewense* Moseley, 1878 is usually much higher than that of the aquatic triclads mentioned above, albeit that this invasive species has established itself outdoors in, for example, several North American states, the West Indies, Portugal, French Guiana, and France (Sluys 2016; Justine et al. 2018). The oxygen consumption of *B. kewense* specimens from outdoor localities in Arkansas, USA was determined at temperatures varying between 27–33 °C, which yielded respiration rates ($\mu\text{LO}_2/\text{g}/\text{WW}/\text{h}$) ranging between 113–290 (Daly and Matthews 1982). It is noteworthy that these values are in the same order of magnitude as those determined for freshwater planarians.

The coefficient *b* based on oxygen consumption of entire specimens of *B. kewense* ranged between 0.686–0.753, as measured at temperatures ranging between 27–33 °C (Daly and Matthews 1982). These values are in the same order of magnitude as those determined for the freshwater triclads *Crenobia alpina* (0.66), *Dugesia gonocephala* (0.82), and *Polycelis felina* (0.82) and the microturbellarians *Mesostoma ehrenbergi* (0.625), *M. lingua* (0.850), and *Opisthomum pallidum* (0.880) (Heitkamp 1979 and references therein).

Terrestrial respiration

Terrestrial flatworms face two problems that involve mutually conflicting adaptations, viz., desiccation and respiration. A cylindrical body, with less surface area:volume ratio, will minimize water loss but restricts diffusion of oxygen to the internal tissues. Probably this is the reason why smaller terrestrial species tend to be round or oval in cross-section, e.g., species of the land planarian genus *Microplana* Vejdovsky, 1890. Therefore, large species tend to be flattened to create a large surface area:volume ratio in order to facilitate diffusion of oxygen to the deep tissues. Nevertheless, the generally large and particularly long species of the land planarian subfamily Bipaliinae Von Graff, 1896 also have a more or less cylindroid body in cross-section.

The partial pressure of oxygen in well-aerated water in equilibrium with air is 0.21 atm (McNeill Alexander 1979) and results in about 9 mgO₂/l at 20 °C. Evidently, the actual amount of dissolved oxygen available at a particular aquatic habitat depends on the temperature, depth, altitude, and the mixing properties of the water (e.g., running water in shallow streams mixing better with air). The amount of oxygen in air is about 30 times that of water (Little 1990; Moore 2006), amounting to about 210 ml O₂, weighing 280 mg, in 1 L of air (Schmidt-Nielsen 1975), while the diffusion rate of oxygen through air is much faster than through water (3 million times faster through air; Prosser 1973).

In the present context it suffices to realize that, thus, availability of sufficient oxygen would not have formed a stumbling-block during an evolutionary transition from water to a terrestrial environment. But, clearly, the organisms needed to evolve respiratory adaptations enabling them to extract oxygen from the air, as opposed to their ancestors, which had evolved in an aquatic habitat.

One such adaptation may be the production of mucus, which is secreted by both aquatic and terrestrial planarians. Mucus plays several important roles in the life of a planarian flatworm and is produced by various kinds of gland. Secretions from glands at the body margin produce a slime trail that facilitates the gliding movement of both aquatic and terrestrial triclads, effectuated by the propulsive force of cilia on the ventral body surface (Jones 1978). Many species of land planarians do not have such dedicated marginal glands, and various types of mucus are secreted from their creeping sole and/or ventral surface. Sticky mucus discharged by cephalic glands partakes in the capture of prey and has neurotoxic properties (cf. Thielicke and Sluys 2019 and references therein). The mucus of triclad flatworms also has repellent properties as the worms are only rarely eaten by other animals since their surface secretions appear to have repugnatorial function (Hyman 1951; Winsor 1998).

As mucus is produced also in aquatic triclads it could well be that these various functions already formed evolutionary preadaptations of similar functions in land planarians. Perhaps the secretion produced by the marginal adhesive zone is an exception as it has been suggested

that it may not provide a lubricant for locomotion in the land planarians but form a moisture-retaining sealant in a resting animal (Winsor 1998). However, one function does not exclude the other.

Production of surface secretions may also have formed a preadaptation for respiration in the terrestrial environment as it covers the body with a “watery” layer that presumably improves the uptake of oxygen. This may be related to the possible role, including respiration, of substances (porphyrins) in rhabdoids that are conspicuous in the dorsal/dorso-lateral epithelium (and microrhabdites over the ventral surface) of land planarians (see above). To the best of my knowledge, this aspect of mucus secretion and respiration in land flatworms has never received any attention.

Evidently, uptake of oxygen through the body wall is only the first step in the respiration process. Hereafter, the oxygen needs to be transported to tissues deeper inside the planarian body. In the absence of respiratory pigments (see above: Respiratory pigments) this can be achieved only by means of diffusion. This implies that also the internal tissues must have a sufficient amount of water in order to be able to dissolve oxygen and carbon dioxide. Maintaining a sufficient level of hydration may be unproblematic for freshwater planarians but may require certain adaptations in marine and terrestrial forms. In particular, land planarians have no physiological or anatomical adaptations for water retention (Kawaguti 1932). On the one hand land planarians have practically no water-saving adaptations, while on the other hand they avoid wet environments and thus are considered to be stenohygic hygrocoeles (Froehlich 1955). It should here be noted that the situation may not be as absolute as suggested by Kawaguti’s (1932) findings, in that land flatworms may prevent desiccation by encasing themselves in mucus. Further, there may be also biochemical adaptations (secondary metabolism; see Campbell 1965) in land planarians for the elimination of carbon dioxide and nitrogenous waste that appear to facilitate water retention, such as the secretion of calcium salts (Percival 1925), which is consistent with being a stenohygic hygrocoele. In contrast to land planarians, freshwater species are subjected to osmotic influx of water and therefore must continuously regulate its volume with the help of their protonephridial system.

One way to assess the hydration of the planarian body is to determine the osmolarity of the tissues, which is expected to vary inversely with the degree of hydration (Jones et al. 2004). The osmolarity of the freshwater planarians *Schmidtea polychroa* and *Girardia dorocephala* (Woodworth, 1897) was 125–128 mOsm kg⁻¹ and 126 mOsm kg⁻¹, respectively, while that of the marine triclad *Procerodes littoralis* (Ström, 1768) ranged between 217–272 mOsm kg⁻¹ (Jones et al. 2004 and references therein). Tissue osmolarity for the terrestrial planarian *Arthurdendyus triangulatus* (Dendy, 1896) ranged between 187.8–257.5 mOsm kg⁻¹, depending upon the laboratory conditions (relatively dry or fully hydrated)

under which the specimens were kept (Jones et al. 2004). These data show that tissue osmolarity in land flatworms is higher than that of freshwater planarians. This means that terrestrial flatworms are less hydrated than freshwater species and that the former are able to maintain a higher osmolarity because they are less prone to influx of water. Evidently, the necessary amount of water that land triclads require for upholding their respiratory physiology is extracted from their frequently, but not always, humid environments. It is a matter of fact that for already millions of years land planarians have successfully evolved the necessary adaptations for exploiting the terrestrial environment but that our understanding of these solutions is still in its infancy.

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